Regional Studies in Marine Science 1 (2015) 1–6

Contents lists available at ScienceDirect

Regional Studies in Marine Science

journal homepage: www.elsevier.com/locate/rsma

The adverse effect of the herbicide atrazine on the reproduction in the intertidal varunid crab *Neohelice granulata* (Dana, 1851)

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HIGHLIGHTS

- We exposed ovigerous females of estuarine crabs to formulated atrazine (2.5, 5 and 15 mg/L) for 32 days.
- Hydropsy and eye atrophy were the abnormalities with higher incidence in hatched larvae.
- Atrazine showed to arrest oocyte growth in the rematurating ovary.

ARTICLE INFO

Article history:
Received 22 October 2014
Received in revised form
17 December 2014
Accepted 21 December 2014
Available online 21 January 2015

Keywords:
Atrazine
Crabs
Larvae
Ovary
Reproduction

ABSTRACT

The effects of a 32-d exposure to atrazine (0, 2.5, 5 and 15 mg/L) were evaluated in the estuarine crab *Neohelice granulata*, both in terms of larvae hatching and ovarian re-maturation. No significant differences ($p > 0.05$) were detected either in incubation time or the number of hatched larvae per female. However, atrazine, particularly at the two higher concentrations, caused several larval abnormalities including hydropsy, hyperpigmented body, atrophy of spines and setae, and atrophy of eyes. At the end of the 32-d exposure period, all assayed atrazine concentrations produced a significant ($p < 0.05$) reabsorption of previtellogenic oocytes, compared to the control treatment. Although no changes in the gonadosomatic index were detected ($p > 0.05$), the proportion of vitellogenic oocytes decreased at all atrazine concentrations, reaching significance at 5 mg/l ($p < 0.05$). These results highlight the potential risk of atrazine for crustacean reproduction, both in terms of altered ovarian maturation and abnormal hatched larvae.

1. Introduction

Atrazine, a herbicide belonging to the triazine group, is one of the most widely used pesticide in the world; although it is a water soluble herbicide, several studies have reported high levels of atrazine in river estuary sediments, with a half life ranging between 2 and 3 months (Graymore et al., 2001). In Argentina, atrazine is mainly applied to crops such as corn and sorghum, with application doses varying between 1 and 2 kg/ha (Atanor, 2012), through an extension of approximately 10 million ha (Arancibia, 2013). During the application period (late spring–early summer), atrazine has been detected in river water at levels rising up to more than 40 μg/L after application; moreover, atrazine was found at concentrations as high as 1 mg/L in waters adjacent to treated fields, as well as in groundwater (Graymore et al., 2001).

The crab *Neohelice* (=*Chasmagnathus*) *granulata* (Decapoda, Brachyura, Varunidae) is one of the most cosmopolitan and conspicuous species widely distributed along the Atlantic coast of South America. In Argentina, this species forms very dense populations along the entire coast of Samborombón Bay, corresponding to the external zone of the "Rio de la Plata" estuary, where the salinity varies from freshwater to almost 30 p.s.u., due to the strong influence of tides (Río de la Plata, 1990). The reproductive season comprises the spring and summer months, when females become ovigerous and migrate off-shore to optimize both larvae hatching and development; the megalopa stage finally returns to the coast, where molting to juvenile instars takes place, to finally reach the reproductive, adult stage (López Greco and Rodríguez, 1999). Both larvae and adult crabs are predated by larvae and adults of several fish, respectively (Menni, 1983; Sanchez et al., 1991).

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http://dx.doi.org/10.1016/j.rsma.2014.12.001
2352-4855/

Samborombón Bay receives the discharge of several rivers and channels that cross extensive agricultural areas, therefore carrying a heavy charge of herbicides, among other pesticides (Río de la Plata, 1990). Particularly, the mouth of Salado River, located at the middle of Samborombón Bay, has been reported as a hot spot of pesticide pollution; in turn, Punta Rasa, the southern edge of the Bay, is considered a clear zone, open to the sea (Río de la Plata, 1990). Atrazine is more intensively applied to both corn and sorghum crops during summer (Thurman et al., 1991), i.e. the reproductive period of *N. granulata.* This species has been previously studied concerning the effects of several pesticides and heavy metals on both embryonic development and larvae hatching (Rodríguez and Pisanó, 1993; Rodríguez and Medesani, 1994; Zapata et al., 2001; Lavolpe et al., 2004; Sanchez et al., 2005; Avigliano et al., 2014). However, little is known about the effects of pesticides on the hormonal regulation of reproduction in *N. granulata* or other crustacean species (Rodríguez et al., 2007; LeBlanc, 2007). Atrazine has shown to act as a xenoestrogen both *in vitro* (Villeneuve et al., 1998; Lascombe et al., 2000) and *in vivo* (McKinlay et al., 2008; Tillit et al., 2010). In crustaceans, atrazine antagonizes the effect of juvenoid hormones (Palma et al., 2009), and alters sexual differentiation in *Daphnia sp* (Dodson et al., 1999). Several reproductive failures, such as a decrease in total *nauplii* production per female, were seen in successive generations of estuarine copepods exposed to environmentally relevant atrazine concentrations (Bejarano and Chandler, 2003).

The main objective of the current study was to evaluate, in the estuarine crab *N. granulata*, the deleterious effects of atrazine, at sublethal concentrations, throughout the egg incubation, larvae hatching and ovarian re-maturation.

2. Materials and methods

N. granulata ovigerous females were collected in October, at the southern edge of Samborombón Bay (35° 56′ S, 57° 06′ W). Only females carrying immature eggs were chosen (mean weight: 9.65 ± 0.47 g, $N = 44$). Once in the laboratory, females were gradually acclimated to the experimental water quality; laboratory conditions (temperature = 25 ± 1 °C, photoperiod = 14:10 L:D, salinity = 30 p.s.u.) reproduced the average values at nature. Semi-static bioassays were run, changing water from all recipients every 72 h. The standard procedures recommended by the American Public Health Association (2005) were followed, also in accordance with the code of ethics for animal experiments stated out by the Declaration of Helsinki.

Stock solutions of atrazine were prepared weekly from Gesaprim 90 WDG® (as soluble granules, 90% w/w, Syngenta Agribusiness) by dissolving the appropriate amount in distilled water. Small aliquots from these stock solutions were added to the test recipients, to achieve a nominal concentration series of 2.5 mg/L, 5 mg/L and 15 mg/L of active principle. These sublethal concentrations were selected after preliminary range-finding test made on adult crabs. Saline water was prepared in all recipients by diluting artificial seawater salts (Tetra Marine Salt Pro, USA) in dechlorinated tap water (hardness: 80 mg/L as equivalents of $CaCO_3$, salinity: 30 p.s.u., pH: 8.0 ± 0.5). Fifteen mL water samples were taken at 0 and 72 h, i.e., the period for water replacement in all test containers, in order to validate nominal concentrations. Samples were filtered through 0.45 μm nylon membrane, and filtrates were analyzed by high liquid pressure chromatography (HPLC) coupled to mass spectrometry (Agilent®, model VL). A X-SELECT C_{18} column was used, using as mobile phase a mixture of acetonitrile:formic acid (0.1%) at 0.5 mL/min. An isotopic tracer of atrazine (^{5}D) was used as a control of analytical quality, while an external standard was used for quantification, at the same conditions used for samples.

2.1. Effects on hatching

Forty four ovigerous females were used (9.41 ± 0.41 g of body weight), assigning 11 females to each atrazine concentration or control. Each female was placed in a glass container filled with 500 mL of the dilution saline water mentioned above, under constant aeration. No food was provided to females during the assay, while mortality, egg loss or hatching larvae were checked daily. Immediately after hatching, two 10-mL samples were taken from each container, stirred to homogenize larvae distribution, and fixed in 5% formalin. The number of hatched larvae per female was estimated by calculating the mean of both samples in relation to the total volume of water in each container. Additionally, for each hatching female a random subsample of 50 larvae was examined under a stereomicroscope, in order to calculate the incidence of each abnormality detected.

2.2. Effects on ovarian re-maturation

Post-hatching females remained exposed to the same atrazine concentrations or control until day 32, counting the beginning of the assay with ovigerous as day 0, under the same experimental conditions above mentioned. However, during this experiment females were fed twice a week on pellets previously used in our laboratory (Chaulet et al., 2012), supplemented with *Elodea* sp. fresh leaves *ad libitum*, in an amount equivalent to 5% of body mass. At the end of the assay, females were weighed to determine body weight (BW) and both ovaries and hepatopancreas were dissected and weighed in order to determine the gonadosomatic (GSI) and hepatosomatic (HSI) index as GSI or HSI = (GW or HW)/BW) × 100, where GW and HW are the gonad and hepatopancreas wet weight, respectively.

Ovaries were then fixed in Bouin solution for 4 h at room temperature, dehydrated in alcohol series and finally embedded in Paraplast, to finally prepare 5-μm sections, stained with hematoxylin and eosin. For each animal, several sections were prepared at different levels of the ovary, and a representative section of each ovary was then analyzed to determine both the relative proportions of normal and abnormal oocytes, as well as oocyte area. Both previtellogenic and vitellogenic oocytes were characterized according to their size and degree of basophilia. To assess the proportions of normal and abnormal oocytes, a grid of 100 points was used in combination with a 40× objective lens. At least three ovarian sections from each animal were examined. Both major (M) and minor (m) diameters of the oocytes showing their nuclei were estimated by means of a micrometric ocular lens, calibrated against a Leitz Wetzlar plate with 1/100 mm spacing; this was used to calculate the oocyte area as $(\pi/4) \times M \times m$ (Rodríguez et al., 1994).

2.3. Statistical analysis

A one-way analysis of variance (ANOVA) followed by least significant difference (LSD) multiple comparisons was used for comparing the experimental groups, concerning incubation time, number of hatched larvae, proportion of each abnormality, proportion of each oocyte type and oocyte area. Logarithmic or angular transformation of data was used when homogeneity of variances was not confirmed in raw data. Percentages (as proportions) of both survival females and females with egg loss were compared between experimental groups by means of the Fisher exact test (Sokal and Rohlf, 1981).

3. Results

Validation of nominal concentrations of atrazine showed a closed correlation with concentrations measured by HPLC ($r^2 = 0.938$ averaging the values at 0 and 72 h, Fig. 1).

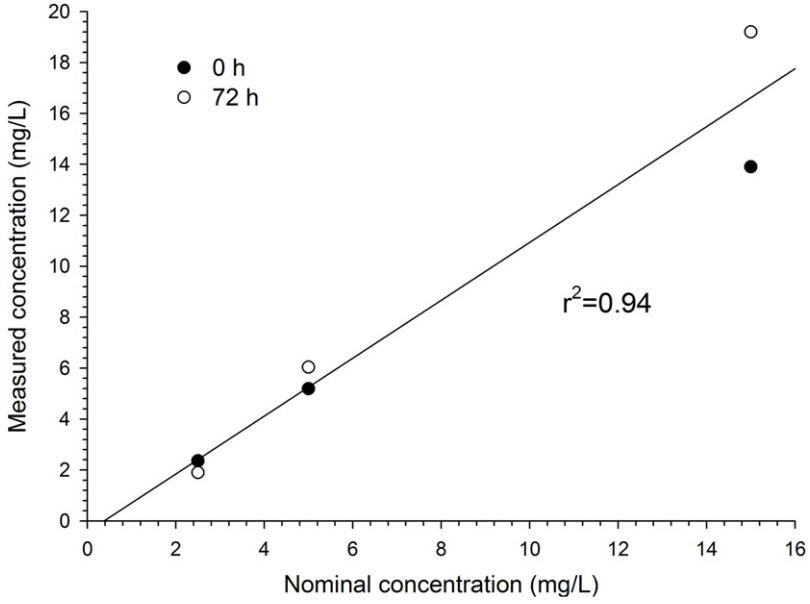

Fig. 1. Nominal concentrations versus concentrations measured at both 0 of the 72 h-water replacement period.

Table 1
Percentage of hatching females, incubation time and number of hatched larvae per females, for each treatment. Mean ± standard errors are expressed.

Atrazine nominal concentration (mg/L)	Number of females	Hatching females (%)	Incubation time (days)	Number of hatched larvae per female
0.0 (control)	11	100.00	9.72 ± 1.28	33745 ± 3551
2.5	11	90.91	11.00 ± 1.07	42459 ± 7940
5.0	11	90.91	9.11 ± 0.93	21032 ± 1799
15.0	11	90.91	10.25 ± 1.39	33072 ± 3634

3.1. Effects on hatching

All control ovigerous females had normal hatching, as well as most of ovigerous females exposed to atrazine; only 1 of 11 females exposed to atrazine had loss of spawned eggs (Table 1). No significant differences ($p > 0.05$) were observed neither in the incubation time nor in the number of hatched larvae per female, for any atrazine concentration compared to control (Table 1).

Several morphological abnormalities were observed in hatched larvae (Fig. 2). Those observed with significant ($p < 0.05$) higher incidence by effect of any atrazine concentrations assayed included: hydropsy, related to an abnormal hydration of tissues, especially in the cephalothorax; atrophy of spines and setae, probably underdeveloped, especially in the maxillipeds; atrophy of eyes, which showed an abnormal contour; hyperpigmented body, showing hypertrophied chromatophores in cephalothorax and pleon, and small body size. Compared to control, there was a significant ($p < 0.05$) higher incidence of hydropsy in all atrazine concentrations, while hyperpigmented body was significantly ($p < 0.05$) increased in the two higher concentrations (Fig. 3). Atrophy of spines and setae, as well as atrophied eyes, were consistently higher than control ($p < 0.05$) at 5 mg/L of atrazine, while a significantly ($p < 0.05$) smaller size was detected at 15 mg/L (Fig. 3).

3.2. Effects on ovarian re-maturation

On day 32nd, mortality in atrazine concentrations ranged between 9 (at 2.5 and 5 mg/L) and 45% (at 15 mg/L), but no significant differences ($p > 0.05$) with control (27%) were detected; number of surviving animals at the end of the assay is shown in Table 2. Neither were significant differences ($p > 0.05$) in GSI and HSI (an overall mean of 0.369 ± 0.033 and 1.470 ± 0.175, respectively, $N = 34$) detected among groups.

Results from histological analysis of ovaries are shown in Table 2. Compared to control, all the atrazine concentrations produced a significant ($p < 0.05$) reabsorption of previtellogenic oocytes. Furthermore, the proportion of normal vitellogenic oocytes decreased in all atrazine concentrations, being significantly ($p < 0.05$) lower than control at 5 mg/L. No significant differences ($p > 0.05$) were noted regarding oocyte area for any oocyte type (Table 2). Prevalence of previtellogenic oocytes in atrazine treated crabs, as well as oocyte reabsorption, can be seen in Fig. 4.

4. Discussion

The lack of information about atrazine levels in water bodies of Buenos Aires province, Argentina, makes it difficult to know the actual herbicide concentration range found in Samborombón Bay. However, atrazine levels detected in surface waters of other countries (Graymore et al., 2001) were, in some cases, relatively close to the lowest concentration employed in the current study. Moreover, atrazine has shown environmental peaks during late spring and early summer (Thurman et al., 1991), in accordance with the application period of this herbicide on crops such as corn, which also coincides with the reproductive period of the studied species (López Greco and Rodríguez, 1999). As a result of this seasonal coincidence, the probability of exposure of *N. granulata* to atrazine is enhanced during a critical period for the survival of the species.

All ovigerous females were alive at the end of the incubation period, confirming that the used atrazine concentrations were sublethal to crabs during that period. For the re-maturation assay,

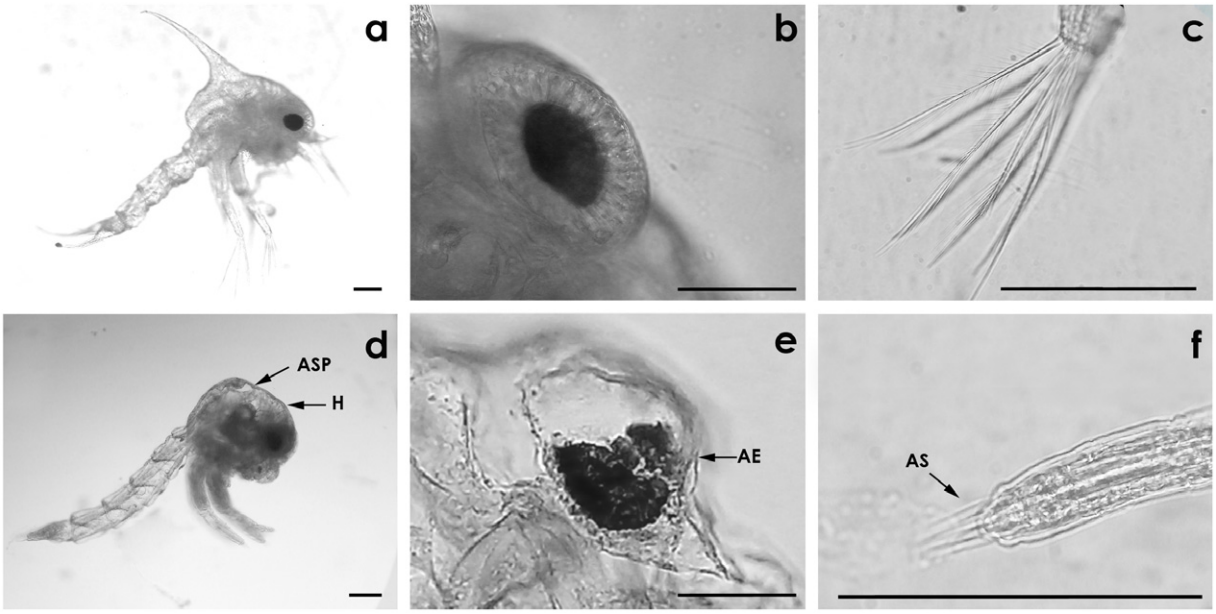

Fig. 2. Abnormalities seen in larvae hatched from ovigerous females of *N. granulata* exposed to atrazine; a: control larvae, b: normal eye, c: normal setae, d: atrophied spines (ASP) and hydropsy (H), e: atrophied eyes (AE), and f: atrophied setae (AS). Scale bar = 10 μm.

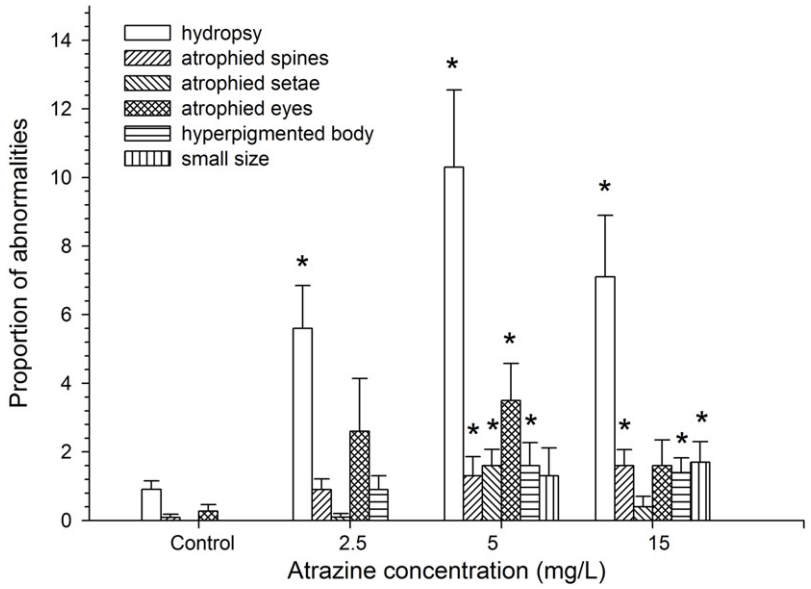

Fig. 3. Proportion of morphological abnormalities in larvae hatched from ovigerous females exposed to atrazine. Mean ± standard errors are indicated. Asterisks indicate significant differences ($p < 0.05$) with respect to control.

Table 2
Percentage of different types of oocyte and oocyte size for each treatment, at the end of the 32-d assay.

Nominal atrazine concentration (mg/L)	Number of females	Pre-vitellogenic oocytes			Vitellogenic oocytes		
		% normal	Area (μm^2)	% reabsorbed	% normal	Area (μm^2)	% reabsorbed
0.0 (control)	8	57.64	1273.64 ± 89.59	0.00	27.51	4243.31 ± 281.30	14.86
2.5	10	53.76	938.72 ± 99.02	21.22*	16.67	3848.81 ± 732.16	8.34
5.0	10	82.42*	1111.33 ± 159.80	16.06*	0.00*	–	1.51
15.0	6	77.82	1026.55 ± 82.15	12.91*	4.28	4084.62 ± 0.00	4.99

* Indicates significant differences ($p < 0.05$) with respect to control.

though, 45% of the females exposed to the highest atrazine concentration died at the end of the 32-d exposure, while only 9% died at the lowest concentration assayed. Despite this trend, none of these percentages was significantly higher than that of control.

Although some pesticides such as parathion and 2,4-dichlorofenoxyacetic acid (Rodríguez and Pisanó, 1993), as well as cadmium (Rodríguez and Medesani, 1994), produced a clear decrement in the number of hatched larvae, current results with atrazine

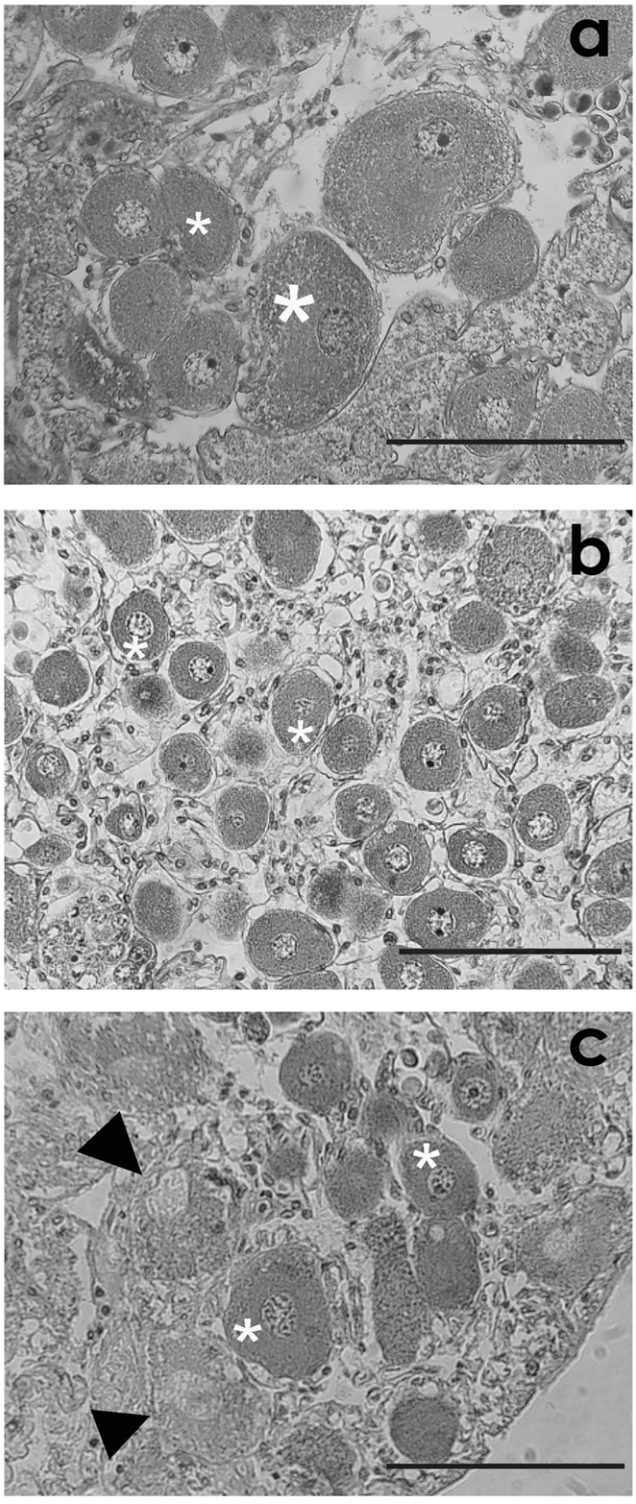

Fig. 4. Histological sections of ovary of *N. granulata*; a: control, with both pre-vitellogenic (*) and vitellogenic (*) oocytes; b: atrazine at 5 mg/L, with only previtellogenic oocytes (*); c: atrazine at 5 mg/L, showing reabsorbed, previtellogenic oocytes (►). Scale bar = 50 μm.

show no changes when compared to control. Hence, no lethal effect of the assayed concentrations of atrazine on developing embryos was evident. However, the effect of this herbicide could be detected in terms of pathologies or abnormalities observed in recently hatched larvae. Although pathologies detected with atrazine were qualitatively similar to those observed after exposing ovigerous females of the studied species to heavy metals such as zinc, lead and mercury (Lavolpe et al., 2004; Sanchez et al., 2005), some abnormalities more frequently detected could be considered an indicator of atrazine exposure. In this regard, hydropsy was the abnormality with highest incidence, followed by eye atrophy.

Hydropsy, namely an abnormal hydration of tissues, could be related to any alteration in osmoregulatory mechanisms, as suggested to other pesticides and heavy metals (Rodríguez and Pisanó, 1993; Rodríguez and Medesani, 1994). Eye atrophy, on the other hand, could have been caused through an alteration of the neural innervations pathway that induces eye formation during embryonic development (Rodríguez et al., 2007). Similarly, larvae from *N. granulata* ovigerous females exposed to parathion (Rodríguez and Pisanó, 1993), a neurotoxic organophosphate insecticide, show severe abnormalities in eye development. Interestingly, atrazine has been cataloged as a potential neurotoxic (Castano et al., 1982; Podda et al., 1997).

In the current study, atrophy of setae and spines, as well as body hyperpigmentation, were detected with lower incidence than either hydropsy or eye atrophy. In the king crab *Lithodes santolla* (Amín et al., 1998), atrophy of setae and spines correlated well with a shorter incubation period, suggesting a possible mechanism for embryos to avoid exposure to pollutants. In the case of *N. granulata* exposed to atrazine, though, no significant differences in incubation time of spawning eggs were seen. Since atrazine is considered as an endocrine disruptor (Rodríguez et al., 2007), this kind of atrophy could be related to altered levels of ecdysone in embryos, as suggested by previous studies exposing *Daphnia magna* to several pesticides (Mu and LeBlanc, 2002). However, no apparent interference of atrazine with ecdysone has been reported in *D. magna* (Palma et al., 2009). Finally, the observed body hyperpigmentation could be the result of both hypertrophy and hyperplasia of normal dark chromatophores, as reported after exposure to several heavy metals for *N. granulata* (Rodríguez and Medesani, 1994; Lavolpe et al., 2004; Sanchez et al., 2005).

Concerning ovarian growth, a clear effect of atrazine on the proportion of different types of oocytes in the ovary was observed, in terms of a lower percentage of oocytes entering to the exogenous (or secondary) vitellogenesis. This effect implies a delay in ovarian maturation, caused by several possible factors. Since no differences were seen by effect of atrazine in GSI, HSI or oocyte area (either for primary or secondary oocytes), there does not seem to be a lower energetic investment in reproduction as a result of the exposure to atrazine. Instead, considering the precedents of atrazine as an endocrine disruptor, some kind of interference of this herbicide with the endocrine system controlling ovarian growth becomes a more plausible hypothesis. Atrazine could be altering different mechanisms. For instance, normal secretion of the gonad inhibiting hormone (GIH) secreted at the eyestalk, which is expected to decrease allowing ovarian maturation (Fingerman, 1997; Nagaraju, 2011), could be increased by atrazine, therefore preventing primary oocytes from developing into secondary ones. Another possibility could be the inhibitory effect of atrazine on the secretion of the gonad-stimulating hormone (GSH) secreted by the thoracic ganglion, leading to delayed ovarian maturation. Additionally, some other possibilities such as the interference of atrazine with the transduction pathway of any hormone acting on the ovary as target organ should not be excluded. In fish chronically exposed to several pesticides, a delay in vitellogenesis has been reported (Mani and Saxena, 1985; Rastogi and Kulshrestha, 1990), possibly due to endocrine disruption (Thomas, 1990).

An increased percentage of oocyte reabsorption was observed in all experimental groups exposed to atrazine. Since in those groups most of oocytes were primary, the augmented reabsorption was only observed in this type of oocyte. This result could be interpreted as a consequence of the possible hormonal disruption exerted by atrazine, leading to an arrested oocyte growth, and/or

as a non-specific response to the stress induced by the chronic exposure to atrazine, as reported by several previous studies concerning multiple stress factors (Wendelaar Bonga, 1997; Maltby, 1999; Power, 2002; Ali et al., 2003). Finally, even when the ovary of females exposed to atrazine could be able to grow, spawning would occur out of the normal reproductive season, i.e., under suboptimal environmental conditions, therefore decreasing the reproductive performance of *N. granulata*.

5. Conclusions

Atrazine was able to produce, at the assayed concentrations (2.5, 5 and 15 mg/L), several abnormalities in larvae hatched from ovigerous females exposed during the egg incubation period; hydropsy and eye atrophy were the abnormalities with higher incidence. Besides, atrazine was shown to arrest the ovarian re-maturation that takes place after spawning, as indicated by a decreased number of oocytes entering to exogenous vitellogenesis, as well as an increased number of previtellogenic oocytes reabsorbed. Our study highlights the potential risk of atrazine for wild crustacean species.

Acknowledgments

This work was supported by the following grants: ANPCYT (PICT2010-0908), UBACYT 2012–2015 (code 044) and PIP CONICET (2010–2012 program, code 100884). We wish to thank Horacio de la Iglesia for helping with the translation.

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